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Age Determination without Tears: Statistical Estimation of Silver Hake (*Merluccius bilinearis*) Age Composition on the Basis of Otolith Weight and Fish Length

Détermination de l'âge sans interruption : Estimation statistique de la composition selon l'âge du merlu argenté (*Merluccius bilinearis*) d'après le poids des otolithes et la longueur du poisson

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ABSTRACT

It is well documented that slow-growing fish produce otoliths that are heavier than fast-growing fish of the same size. As a result, the analysis of combined otolith weight and fish length measurements is better able to distinguish among age groups than is either variable on its own. Here the accuracy of a statistical model for estimating the proportions at age in an unknown sample is tested by taking advantage of the relationship between otolith weight, fish length, and age in a calibration sample. The accuracy of statistically-estimated age proportions in research vessel samples of silver hake (*Merluccius bilinearis*) was reasonably good, and on the order of that expected of inter-reader age variability. If a suitably large subsample of silver hake from a given fishery and sex were aged, and then used as a reference for estimating the age composition of the remaining fish of the same groups, the results are very likely to be acceptable to the stock assessment. Based on results to date, it also appears that recently-aged years could be used to estimate the age composition of an unknown recent year with acceptable accuracy. On the other hand, continuous use of the statistical method for estimating age composition on a routine annual basis (across many years) would be inadvisable without periodic calibration.

RÉSUMÉ

Grâce à une bonne documentation, nous savons que les poissons dont la croissance est lente produisent des otolithes plus lourds que les poissons à croissance rapide de la même taille. Par conséquent, l'analyse des mesures combinées du poids des otolithes et de la longueur du poisson permet de mieux établir la distinction parmi les groupes d'âge que l'une de ces deux mesures seule. Dans ce cas, la précision d'un modèle statistique pour l'estimation des proportions à un certain âge dans un échantillon inconnu est mise à l'essai en tirant profit de la relation entre le poids des otolithes, la longueur du poisson et l'âge dans un échantillon d'étalonnage. La précision des proportions selon l'âge estimées statistiquement dans les échantillons de merlu argenté (*Merluccius bilinearis*) prélevés par les navires de recherche était relativement bonne et dans la fourchette prévue de la variabilité de l'âge selon plusieurs appareils de lecture. Si l'âge d'un sous-échantillon suffisamment grand de merlus argentés issus d'une pêche donnée ou avec un âge donné était établi, puis utilisé en tant que référence pour l'estimation de la composition selon l'âge des poissons restants des mêmes groupes, il est fort probable que les résultats seraient acceptables pour l'évaluation du stock. D'après les résultats obtenus à ce jour, il semblerait également que les années pour lesquelles l'âge a été établi récemment pourraient être utilisées pour estimer la composition selon l'âge d'une année récente inconnue avec une précision acceptable. En revanche, l'utilisation continue de la méthode statistique pour l'estimation de la composition selon l'âge chaque année (pendant de nombreuses années) serait à déconseiller sans un étalonnage périodique.

INTRODUCTION

Around the world, the ages of close to a million fish are determined each year using otoliths, largely in support of harvest calculations (Campana and Thorrold 2001). Fish age is generally determined after initial preparation of the otolith (such as embedding and thin sectioning), followed by microscopic examination and counts of the annual growth zones (annuli). The preparation process is often time consuming, while the interpretation of the annuli requires skilled technicians. As a result, the process of age determination is reasonably expensive. To minimize time and expense, many agencies take small subsamples of catches or populations for age estimates, producing age-length keys that are used to infer the age composition of the remainder of the catch based on a larger sample of simple length measurements (Kimura 1977).

While age-length keys rely on the relationship between age and fish length, they can only be applied to length frequencies from the same time period and fishery. An alternative approach, and one which avoids this constraint, is to take advantage of the well-documented proportionality between the size of the otolith and both the size and age of the fish (Templeman and Squires 1956). Although the size of the fish and the otolith are correlated, otolith size tends to be somewhat more correlated with fish age than is fish length (Boehlert 1985). Thus in principle, otolith size can better be used to infer fish age than can fish length. A number of studies have statistically related various measurements of otolith size (e.g., otolith weight, length, area) to the annulus-based age, and then used the resulting relationships to estimate the age composition of the remaining, unaged fish (Boehlert 1985; Pawson 1990; Fletcher 1995; Worthington et al. 1995). A common feature shared by this approach and that of the age-length key is that both require two samples: a "calibration" and a "production" sample. The calibration sample (sometimes called the reference or training sample) is used to define a procedure for estimating age, and this procedure is then applied to all fish in the production sample (sometimes called the unknown or test sample). The ages of fish are known in the calibration sample but not in the production sample. The motivation for this two-stage approach is simple – the first stage involves expensive annulus-based age determinations, while the second stage does not.

A key point to remember is that the most common product of age determinations is that of catch proportions at age for use in stock assessments. The second most common output would be growth parameters. Thus, the goal is nearly always to estimate the growth or mortality parameters of a fish population, not to estimate the ages of individual fish (Pauly 1987). Although otolith measurements do a relatively poor job of assigning ages to individual fish, the same suite of measurements can often be used to provide more accurate estimates of population parameters. This approach has been reviewed by Francis and Campana (2004).

In this research document, a statistical model is presented for estimating the proportions at age in an unknown sample by taking advantage of the information in a calibration (aged) sample. Because the statistical method is based on maximum likelihood, it avoids the asymptotic bias that characterizes other methods. It is concluded with an application of the method to silver hake, and it discusses the potential of the method for generating catch at age data in future stock assessments.

BIOLOGICAL BASIS

Otolith size (both length and weight) is roughly proportional to the size of the fish. Therefore, otolith size by itself can give some idea of the age of the fish, in the same way that fish size is a rough approximation of fish age. For young fish, fish length and otolith size modes may be discrete enough that age can be inferred through a simple mixture analysis (based on separation of normally distributed mixtures). However, growth rate slows with age, so the fish length and otolith size distributions soon become unresolvable.

It has been well documented that slow-growing fish have relatively large otoliths compared to faster-growing fish. As a result, in two fish of identical length, the older of the two fish will have a larger otolith. This difference is usually statistically significant and is common to most species. There is a physiological basis for this effect, so it is consistent (Figure 1).

Measurements of known age fish have demonstrated that fish of a given age can be described as some function of fish length and otolith weight (Figure 2). Conceptually, this can be considered as a bivariate ellipse, with a normal error distribution around each variable, and covariance between the two variables. Statistical estimation of this age-specific ellipse is the basis for the method described here.

THE MODEL

The model described here estimates the age group proportions in an unknown sample of fish based on otolith weight and fish length measurements available in a calibration sample. Although the reference sample is assumed to have a similar relationship between fish length and otolith weight as is present in the unknown sample, four variants of the model allow for differences between calibration and unknown samples.

For both reference and unknown sample, the data for the age group estimation consists of a vector of variables x. For the purpose of this study, these variables will include fish length and otolith weight, but could also include otolith length and other variables. A within age group normal distribution is assumed for the variables: the x for a randomly selected fish from age group g is normal with mean vector μ_g and covariance matrix Σg. The distribution of a fish for which the age group is not known is then a mixture of normals.

Let x_i denote the vector for the ith fish in the reference sample and let a_i denote its age. Then the likelihood contribution for a fish from the reference sample is:

$$f(x_i; \mu_{ai}, \Sigma a_i)$$

where $f(\cdot; \mu, \Sigma)$ is being used to denote the density of a multivariate normal distribution with mean vector μ and covariance matrix Σ. To simplify notation, one can think of all of the means, variances, and covariances for all of the different age groups being aggregated into a single parameter vector ζ. The likelihood contribution, for all of the fish in the reference sample is then:

$$L_r(\zeta) = \prod_i f(x_i; \mu_{a_i}, \Sigma_{a_i})$$

where the product is over all fish in the reference sample.

For the unknown sample, the age groups are unknown. The likelihood contribution for the ith of these fish is a mixture or weighted average of the normal distributions for the different age groups

$$f(\mathbf{x}_i;\zeta,\pi)=\sum_a \pi_a f(\mathbf{x}_i;\mu_a,\Sigma_a)$$

where the sum is over all age groups and π_a is the probability that a randomly selected fish is from the ath age group. The vector π is the quantity of interest; if simple random sampling is done, π_a represents the proportion of fish in the ath age group in the population that the unknown sample was drawn from. The likelihood contribution, $L_m(\zeta, \pi)$, for all of the fish in the unknown sample is then:

$$L_m(\zeta,\pi)=\prod_i f(\mathbf{x}_i;\zeta,\pi)$$

where the product is over all fish in the unknown sample. The full likelihood for all of the data is the product of the likelihood contributions for the reference and unknown samples:

$$L_f(\zeta, \pi)= L_m(\zeta, \pi)L_r(\zeta)$$

Three different fitting procedures are considered. In each case, the E-M algorithm (Dempster et al. 1977) is used to obtain the fitted parameters.

1. Full maximum likelihood estimation (Omixf). The full likelihood $L_f(\zeta, \pi)$ is maximized. In theory, this is *the* appropriate methodology since it most fully uses the data. Practical concerns about outlying values in the unknown sample or the representativeness of the reference sample often makes it useful to consider alternatives.

2. Maximum likelihood estimation of π using the unknown sample (Omixr). Here the within age group covariance matrices and mean vectors, ζ are either input by the user or are estimated from the reference sample. These are then substituted into the mixed likelihood as if they were the true age group means and covariances. The mixed likelihood $L_m(\zeta, \pi)$ is then be maximized over all possible age group probabilities π treating ζ as fixed. If the reference sample gives a good representation of the data in the unknown sample and there are large numbers of fish in it, then this methodology might be desirable since it is less influenced by outlying values in the unknown sample.

3. Maximum likelihood estimation using the reference sample to obtain a starting ζ (Omixs). Here, sample means and covariance matrices are either input by the user or are calculated from the reference sample and are used only to obtain starting values. The mixed likelihood $L_m(\zeta, \pi)$ is then maximized over all possible age group probabilities π and over part or all of the parameters in ζ. In the case that the data in the reference sample is not representative of the unknown sample, this would be the appropriate methodology to use since it estimates means and (optionally) covariance matrices from the unknown sample alone. Omixs-a determines covariance matrices that maximize the likelihood for the unknown data, while Omixs-b fixes the within age group covariance matrices (but not the means) at those of the reference sample.

THE DATA

Silver hake samples were those from the research vessel (RV) only, so as not to confound RV and commercial growth rates. For most analyses, only ages from female silver hake were used, so as to avoid complications due to sex-specific growth rates. All otolith ages were derived from a single age reader viewing intact otoliths under a binocular microscope using reflected light after several months of clearing in a glycerin:water mixture. Otolith weights to the nearest milligram were obtained after removal of all surface glycerin.

Replicate age readings were available for one of the sample years (1990), and the resulting precision was assumed to be representative of more recent ageing.

Ages were available for both reference and unknown samples, thus allowing comparison between estimated age proportions and those that were actually calculated. However, unavoidable random ageing error between replicate age readings implies that the difference between calculated and estimated age proportions could be smaller or larger than calculated.

RESULTS

Both fish length and otolith weight increased monotonically with age, with the difference between age groups declining in older fish (Figure 3). Fish length- and otolith weight at age were relatively similar among years for most of the younger age groups; however, significant differences in size at age were evident for older fish from the 1990 and 2008 samples relative to the other years. These differences persisted even if the analysis was restricted to female silver hake (Figure 4).

There were clear and significant differences in growth rate and longevity between male and female silver hake, with females growing to older ages and greater lengths than males (Figure 5). Similar sex-specific differences were apparent with respect to otolith weight, but of smaller magnitude.

The relationship between fish length and otolith weight was exponential (Figure 6). Age groups tended to be clearly segregated at ages less than about 4, with increasing overlap at greater ages. The variance between fish length and otolith weight was less marked when restricted to females.

Before the accuracy of the statistical age estimation method can be appraised, it is useful to see the variability in age composition due only to random and unavoidable otolith ageing error. Figure 7 shows the observed age proportions in a 1990 RV sample after a single age reading, and then after replacing those ages with a replicate set of ages by the same age reader. The coefficient of variation (CV) between these two sets of age readings was 2%, which is considerably more precise than would normally be expected in replicate age readings of silver hake (Campana 2001). The calculated age proportions were similar between the two sets of age readings, but differed by up to 4% at any given age (Table 1). The mean absolute deviation, summed across ages 1-8, was 1.6%. These results provide a rough guideline for interpreting the estimated age proportions that follow, since they indicate that age differences of at least 4% fall within the bounds of normal ageing variability.

As an initial test of the relative accuracy of the statistical ageing method, the age composition of the 2009 sample was estimated using the 2008-2010 samples as the pooled reference sample

(Figure 8). Note that the unknown sample was actually a subsample of the reference sample (making up about one third of it), thus making this example a "best case" scenario. The estimated age proportions were quite similar to those that were actually present, particularly for the Omixs-a model. The mean absolute deviation, summed across ages 1-9, was 1.1%, which was slightly better than the deviation expected of repeated age readings.

More realistic tests of the age estimation models involved the use of unknown samples that were completely independent of the reference samples. Age proportions in 2011 were estimated using 2008-2010 samples as reference, resulting in a reasonably good set of predictions using Omixr (Table 2; Figure 9). The mean absolute deviation of this model was 2.2%. As a second test, the 2008 age proportions were estimated using the 2009-2011 data as reference (Table 2; Figure 9). In this test, Omixs-b provided the best fit, with a mean absolute deviation of 1.9%.

To test the limits of the prediction model, the 1990 age proportions were estimated using 2008-2010 as the reference set, years which differed significantly from 1990 in mean fish and/or otolith size at age for some ages. Since the estimation method assumes that the reference set is similar to the unknown set, this particular test strays outside acceptable boundaries. Nevertheless, all but one of the age proportions was well estimated (Table 3; Figure 10). Once again, model Omixs-a provided the best fit, with a mean absolute deviation of 4.4%.

DISCUSSION

Both the biological and the statistical bases of the age estimation approach described in this study are well founded. The observation that slow-growing fish produce otoliths that are heavier than fast-growing fish of the same size has been noted in numerous species, including in known-age fish (Templeman and Squires 1956; Cardinale et al. 2004). Given that otoliths tend to grow even in fish that have stopped growing in length, one would expect to see age-specific differences in the fish-otolith relationship. An unbiased statistical basis for this approach has also been worked out and verified (Francis et al. 2004; Francis et al. 2005). What has been lacking to this point is a proper test of the method using real fish.

In the tests using totally independent reference and unknown data sets, the accuracy of the statistically-estimated age proportions was reasonably good, and on the order of that expected of inter-reader age variability. Random ageing variability is expected of any set of age determinations, whether based on otoliths or other calcified structures. Precision (measured as CV) on the order of 5% is expected of all fish ageing studies, although gadid age precisions of 2-4% are not uncommon (Campana 2001). Only one set of repeated ages was available for silver hake, so it is not clear if the CV of 2% that was measured was typical, or was unusually precise. Assuming the latter, it may be that the statistically-estimated age proportions are even better than are indicated, since not all of the observed ages are necessarily correct. In the absence of truly known-age silver hake, this cannot be tested.

After repeated testing of the age estimation models, several characteristics and constraints of the models became evident. Firstly, the prediction accuracy of the models improved substantially with sample size. Prediction accuracy was not as good when only one year was used ($n<150$) as a reference; a sample size of about 500 fish appeared to strike a good balance between sample size and practicality. Secondly, prediction accuracy was sensitive to the similarity in growth characteristics between reference and unknown fish. This became evident in predicting the age composition of the 1990 sample using 2008-2010 fish as reference. Since fish/otolith growth similarity between reference and unknown is an underlying assumption of the

prediction model, this finding was expected. This implies that similar years should be used to calibrate the unknown year, or ideally, a subsample of the same year. It also suggests that RV otoliths should not be used to estimate the age composition of commercial samples, and vice versa. Finally, it was not always clear which model variant would produce the most accurate age composition predictions. In general, Omixr and Omixf did not perform as well as the two variants of Omixs. Nevertheless, there were some trials where Omixs did not produce the most accurate results. Further research may be required to address this issue.

Although there are numerous fish species for which routine age determinations are used in support of stock assessment, silver hake is an ideal candidate for statistical estimation of age composition. The species is reasonably fast growing with relatively few age classes. However, unlike some other species, growth rate is sexually dimorphic, which required separate age estimation for each sex. Nevertheless, this is a tractable problem.

If the statistical age estimation method were to be adopted for use, there are several steps that could be taken to readily improve the accuracy of the results. One such step would be to have the age reader re-age one or more years of RV samples, so as to acquire better estimates of the true variance around the observed age composition. If the actual age composition is more imprecise than was calculated based on the one set of replicate age readings, this would imply that the statistical method is somewhat better than current results indicate. Secondly, it would be better to record otolith weights before adding glycerin for clearing, and thus avoid an unnecessary source of weighing error.

Is the statistical method for estimating age composition ready for routine use in silver hake? The answer is 'in some cases'. If a suitably large subsample of fish from a given fishery and sex were aged, and then used as a reference for estimating the age composition of the remaining fish of the same groups, the results are very likely to be acceptable. Based on results to date, it also appears that recently-aged years could be used to estimate the age composition of an unknown recent year with acceptable accuracy. This type of approach is not possible using conventional age-length keys. On the other hand, continuous use of the statistical method for estimating age composition on a routine annual basis (across many years) would be inadvisable without periodic calibration.

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Table 1. Variation in proportion at age in a sample of silver hake (n=113) due solely to inter-reader variability (CV=2%) in age determination.

	Proportions by Age								
	Age 0	Age 1	Age 2	Age 3	Age 4	Age 5	Age 6	Age 7	Age 8
Actual - 1st set of ages	0.000	0.195	0.186	0.062	0.044	0.027	0.248	0.168	0.071
Actual - 2nd set of ages	0.000	0.186	0.204	0.053	0.044	0.062	0.221	0.159	0.053
Difference	0.00	0.01	-0.02	0.01	0.00	-0.04	0.03	0.01	0.02

Table 2. Estimates of the 2009 female silver hake proportion at age (n=263) using four model variants and various aged samples as reference (Predictor).

			Proportions by Age								
Unknown	Model	Predictor	Age 1	Age 2	Age 3	Age 4	Age 5	Age 6	Age 7	Age 8	Age 9
2009 female	Actual		0.232	0.152	0.179	0.175	0.099	0.084	0.046	0.019	0.015
	Omixr	2008	0.209	0.200	0.214	0.013	0.306	0.017	0.039	0.000	0.000
	Omixs-a		0.208	0.241	0.067	0.100	0.135	0.195	0.007	0.044	0.000
	Omixs-b		0.181	0.188	0.265	0.075	0.262	0.016	0.007	0.005	0.000
	Omixf		0.209	0.196	0.222	0.000	0.323	0.018	0.030	0.000	0.000
	Omixr	2009	0.228	0.152	0.171	0.204	0.066	0.136	0.009	0.013	0.018
	Omixs-a		0.225	0.159	0.185	0.159	0.119	0.072	0.058	0.007	0.014
	Omixs-b		0.226	0.149	0.205	0.236	0.075	0.004	0.086	0.004	0.011
	Omixf		0.226	0.153	0.173	0.211	0.056	0.147	0.000	0.012	0.018
	Omixr	2010	0.225	0.176	0.219	0.041	0.317	0.000	0.020	0.000	0.000
	Omixs-a		0.224	0.159	0.246	0.208	0.007	0.126	0.027	0.000	0.000
	Omixs-b		0.225	0.173	0.234	0.209	0.006	0.142	0.009	0.000	0.000
	Omixf		0.224	0.176	0.207	0.140	0.114	0.112	0.024	0.000	0.000
	Omixr	1990	0.199	0.095	0.344	0.059	0.298	0.001	0.001	0.001	0.000
	Omixs-a		0.203	0.125	0.265	0.095	0.201	0.061	0.040	0.007	0.000
	Omixs-b		0.136	0.101	0.333	0.052	0.083	0.003	0.284	0.004	0.000
	Omixf		0.219	0.097	0.277	0.078	0.269	0.042	0.000	0.014	0.000
	Omixr	2008-2010	0.222	0.175	0.212	0.080	0.201	0.075	0.000	0.017	0.014
	Omixs-a		0.222	0.174	0.188	0.151	0.111	0.079	0.051	0.007	0.012
	Omixs-b		0.219	0.177	0.243	0.213	0.126	0.000	0.006	0.008	0.004
	Omixf		0.221	0.176	0.212	0.085	0.191	0.082	0.000	0.015	0.014

Table 3. Estimates of female silver hake proportion at age from different years using four model variants and various aged samples as reference (Predictor).

			Proportions by Age								
Unknown	**Model**	**Predictor**	**Age 1**	**Age 2**	**Age 3**	**Age 4**	**Age 5**	**Age 6**	**Age 7**	**Age 8**	**Age 9**
1990 female	**Actual**		**0.195**	**0.186**	**0.062**	**0.044**	**0.027**	**0.248**	**0.168**	**0.071**	**0.000**
	Omixr	2008-2010	0.208	0.219	0.027	0.000	0.000	0.000	0.374	0.115	0.055
	Omixs-a		0.190	0.233	0.033	0.036	0.033	0.233	0.025	0.100	0.110
	Omixs-b		0.205	0.219	0.061	0.001	0.301	0.087	0.011	0.000	0.112
	Omixf		0.206	0.220	0.030	0.034	0.000	0.000	0.000	0.196	0.311
2011 female	**Actual**		**0.149**	**0.281**	**0.132**	**0.153**	**0.096**	**0.089**	**0.064**	**0.036**	**0.000**
	Omixr	2008-2010	0.162	0.286	0.123	0.186	0.146	0.056	0.027	0.030	0.000
	Omixs-a		0.122	0.314	0.091	0.246	0.044	0.070	0.025	0.067	0.020
	Omixs-b		0.160	0.278	0.039	0.261	0.142	0.030	0.000	0.000	0.087
	Omixf		0.160	0.268	0.123	0.183	0.174	0.018	0.029	0.041	0.000
2008 female	**Actual**		**0.199**	**0.265**	**0.226**	**0.133**	**0.093**	**0.040**	**0.031**	**0.013**	**0.000**
	Omixr	2009-2011	0.188	0.265	0.202	0.208	0.000	0.035	0.059	0.035	0.005
	Omixs-a		0.156	0.308	0.062	0.279	0.063	0.049	0.024	0.024	0.031
	Omixs-b		0.187	0.272	0.264	0.109	0.100	0.000	0.057	0.004	0.006
	Omixf		0.186	0.268	0.194	0.226	0.000	0.000	0.091	0.029	0.001

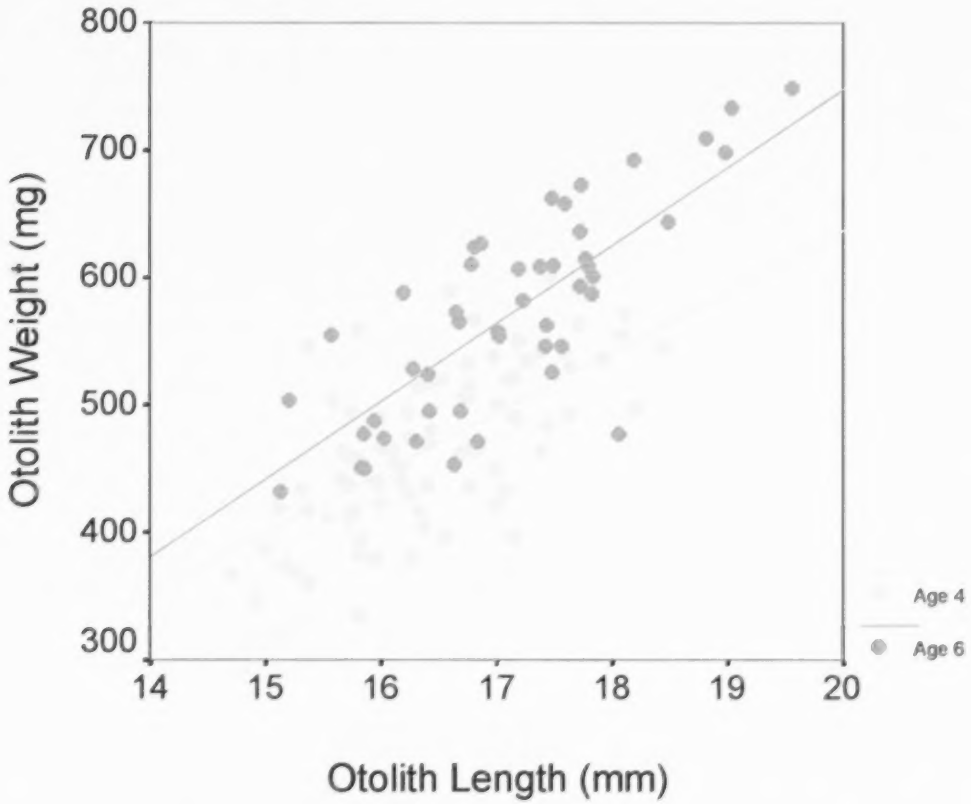

Figure 1. Relationship between otolith weight and otolith length in wild cod of truly-known age. Older fish of a given length have heavier otoliths than do younger fish of the same length. A similar relationship exists between otolith weight and fish length.

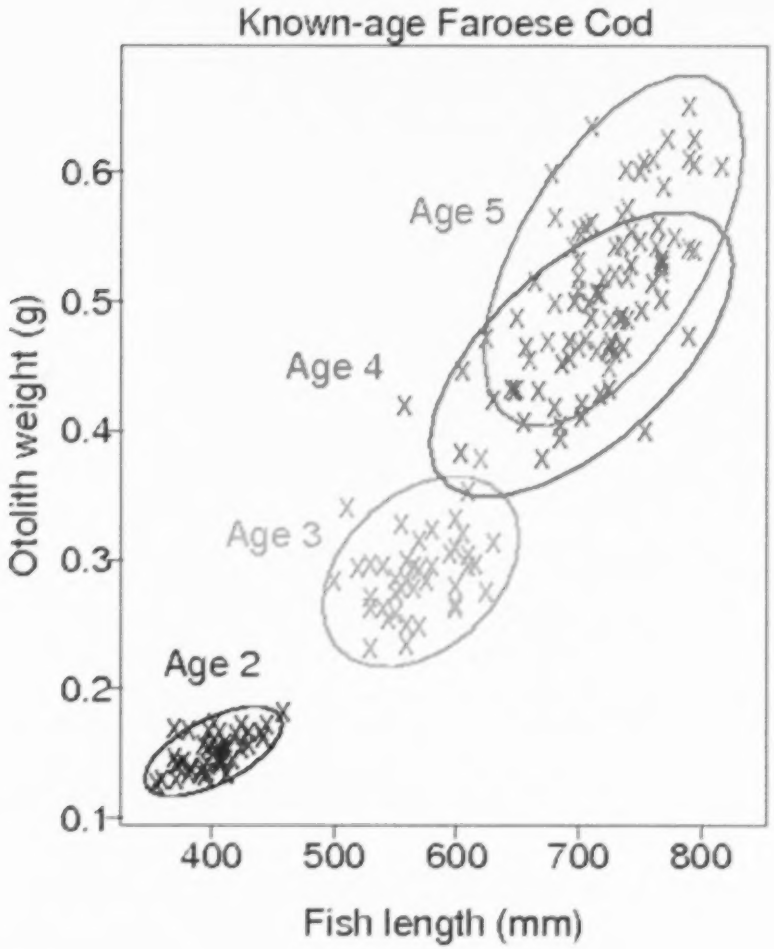

Figure 2. Age-specific relationship between otolith weight and fish length in wild cod of truly-known age. A bivariate ellipse can be used to describe the relationship for each age group. This ellipse can be estimated statistically using four variants of a maximum likelihood-based method.

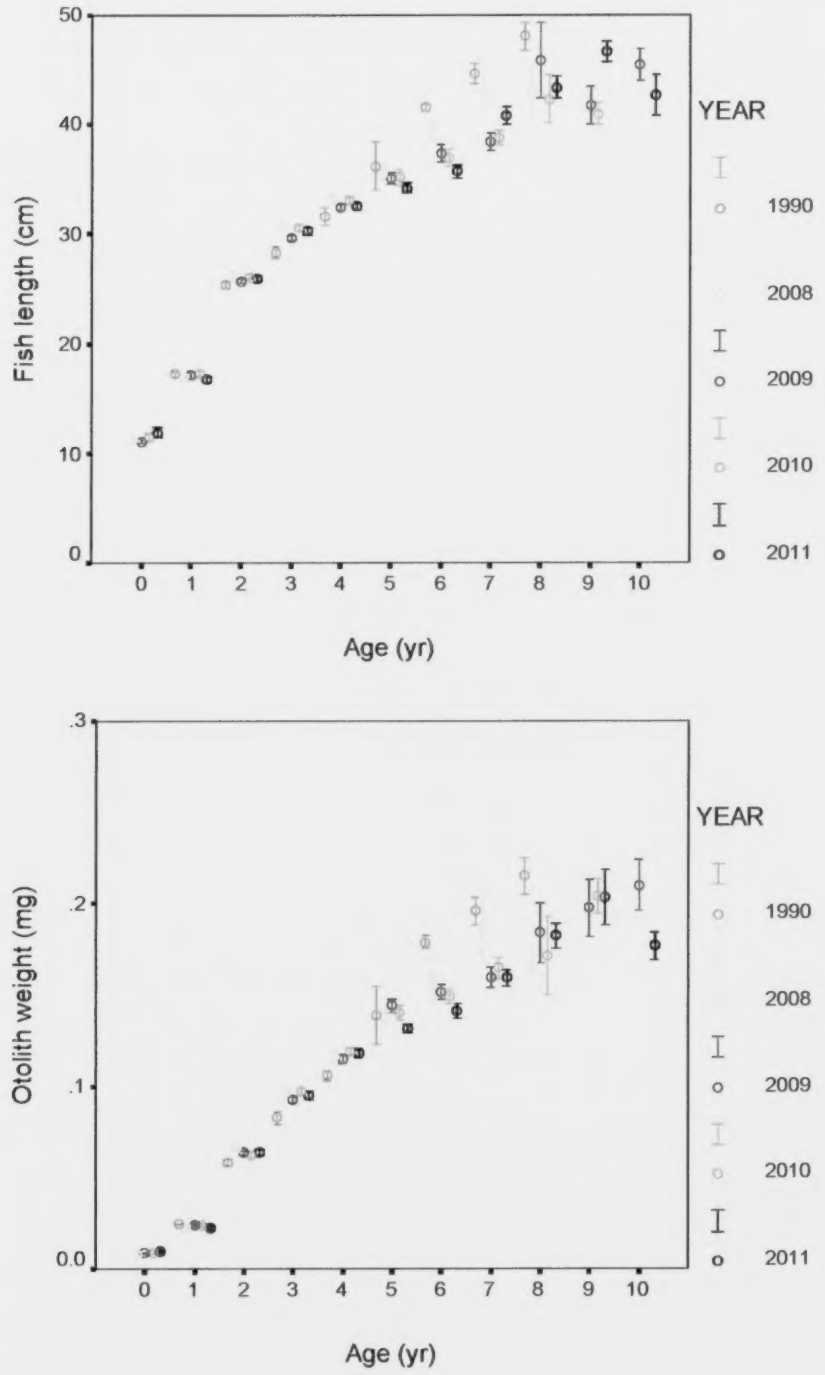

Figure 3. Inter-annual variations in fish length (top) and otolith weight (bottom) in silver hake from the research survey.

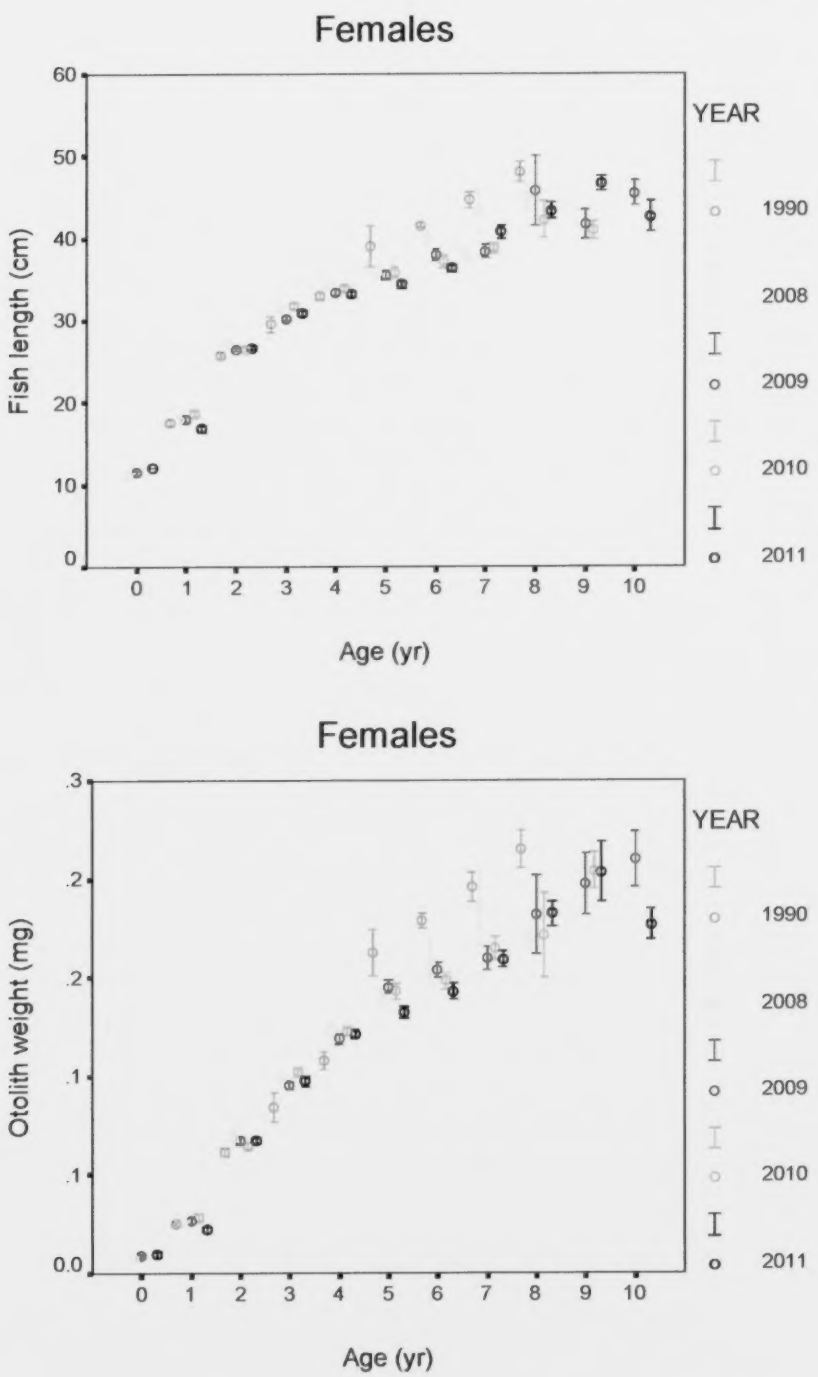

Figure 4. Inter-annual variations in fish length (top) and otolith weight (bottom) in female silver hake from the research survey.

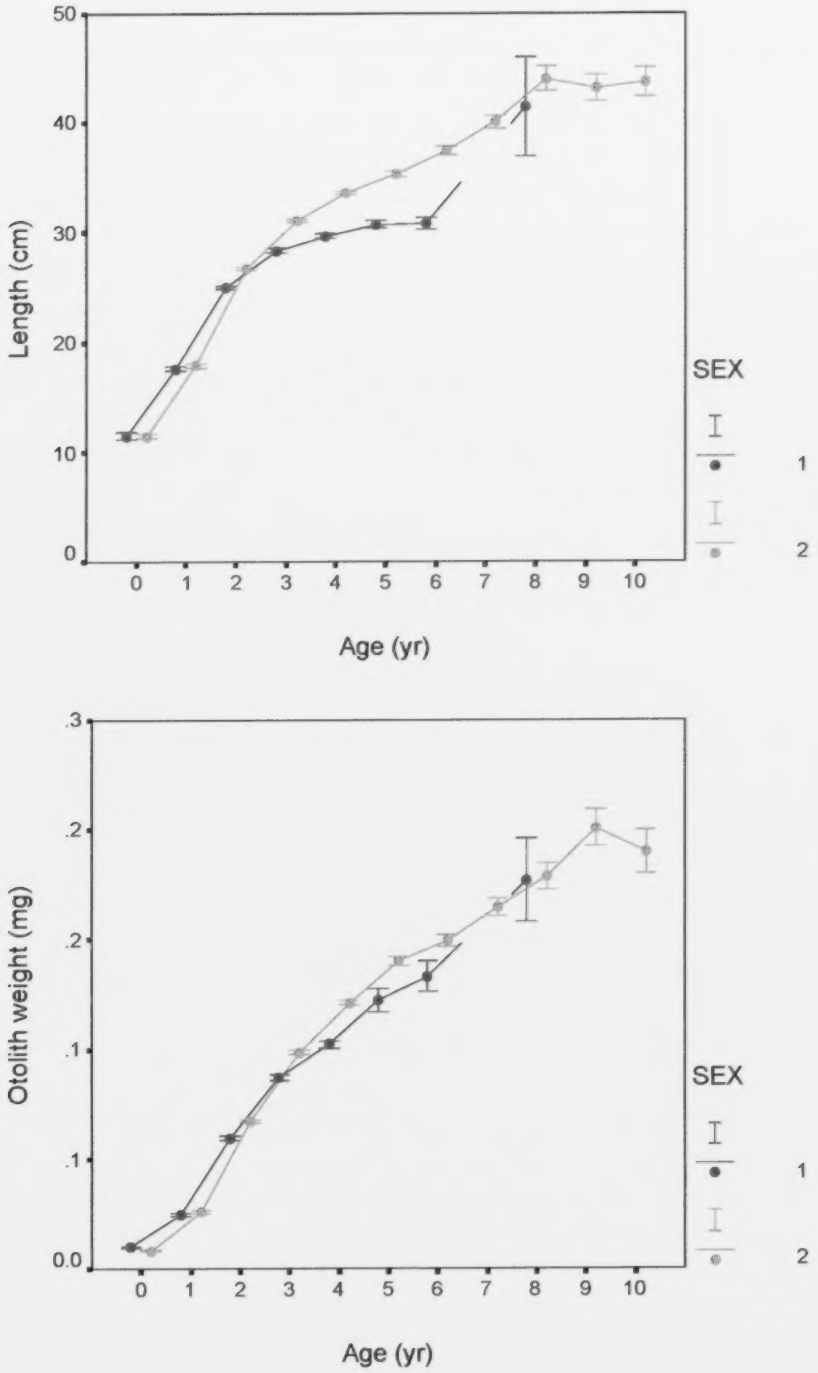

Figure 5. Sex-specific size at age variations in fish length (top) and otolith weight (bottom) in silver hake from one year of the research survey. Males are coded as 1, females as 2.

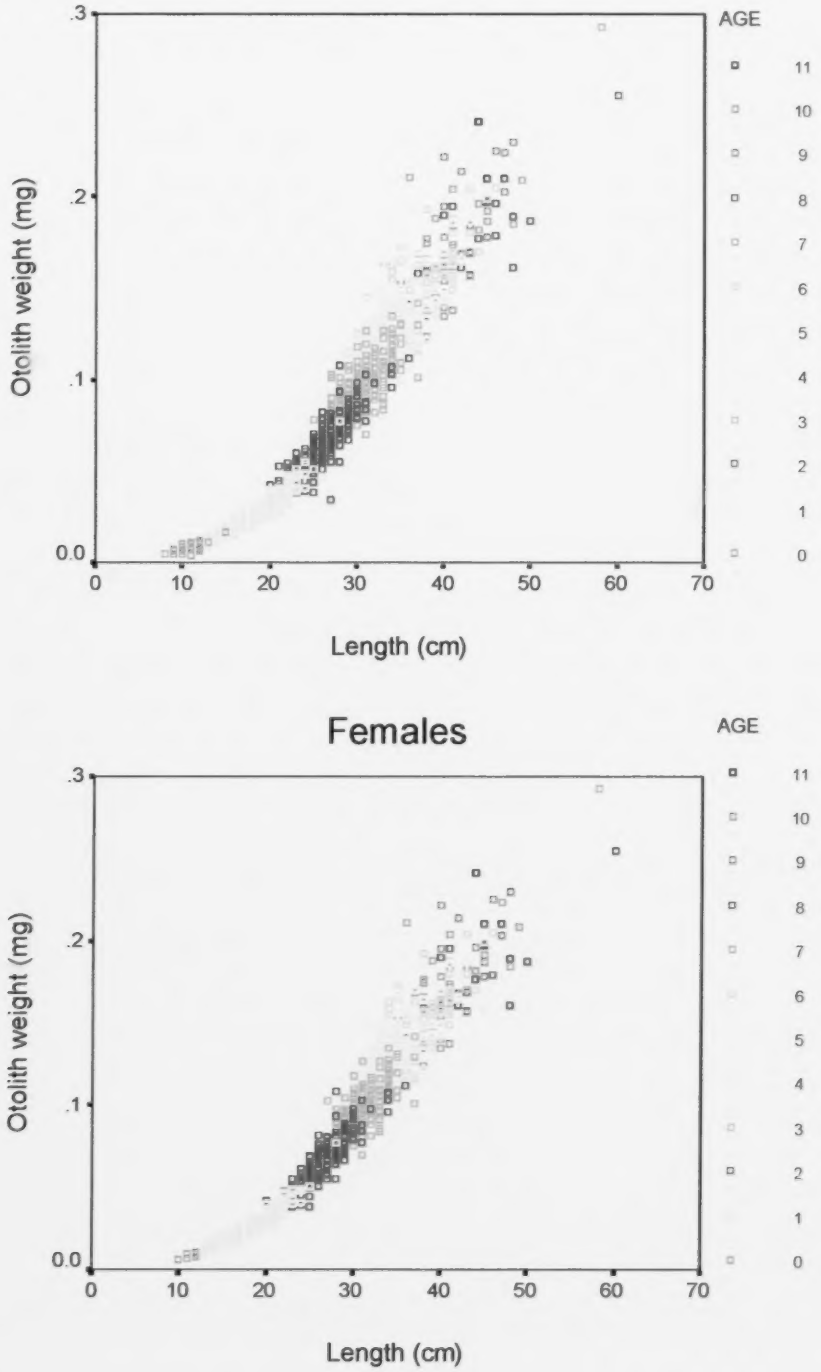

Figure 6. Age-specific relationship between fish length and otolith weight in silver hake, both for sexes combined (top) and for females only (bottom).

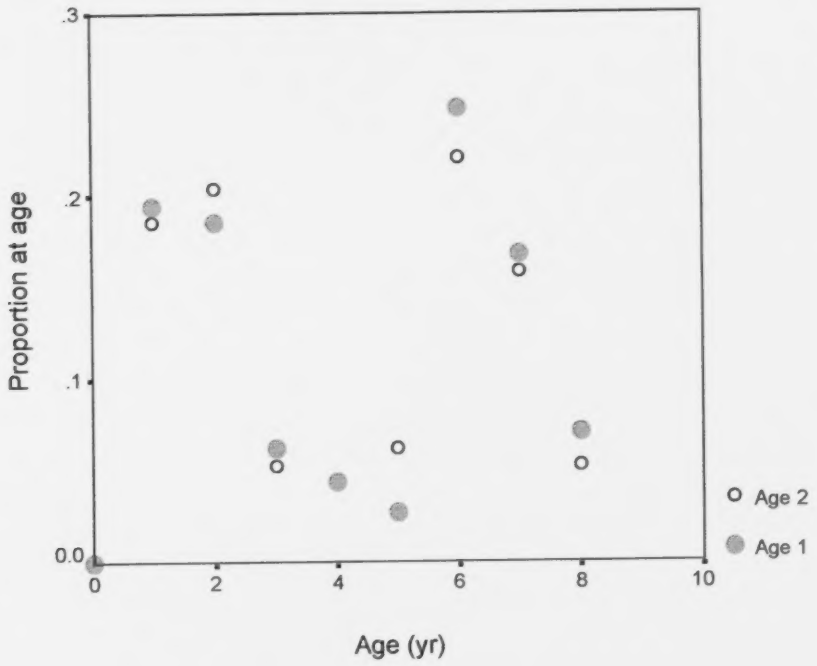

Figure 7. Differences in the calculated proportion at age in a sample of silver hake due solely to variability between independent age readings.

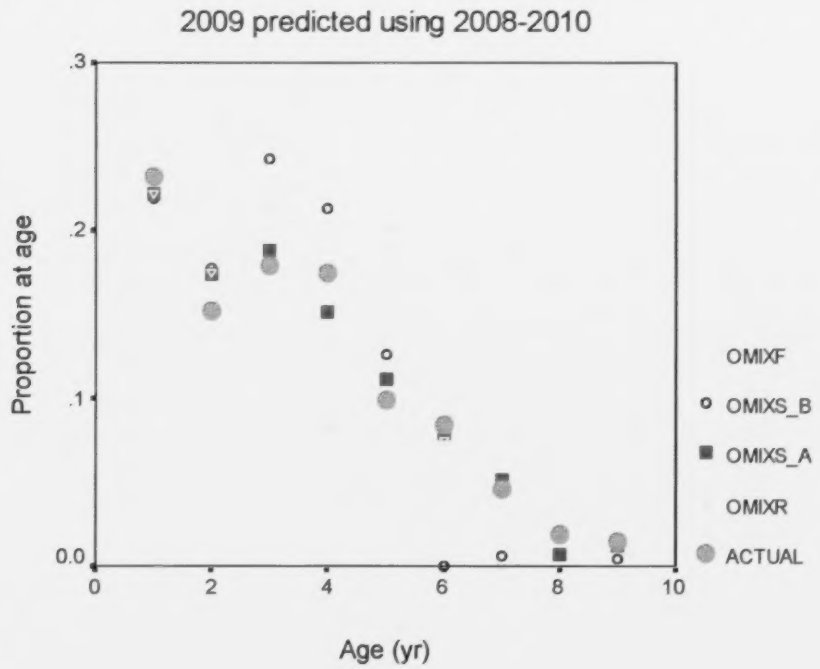

Figure 8. Proportion at age in the 2009 research vessel silver hake collection (n=263; red symbols) compared to estimates derived from four predictive models based on 2008-2010 data (n=729). Note that the year being estimated comprises a portion of the reference data. The model providing the most accurate estimates is indicated by blue squares.

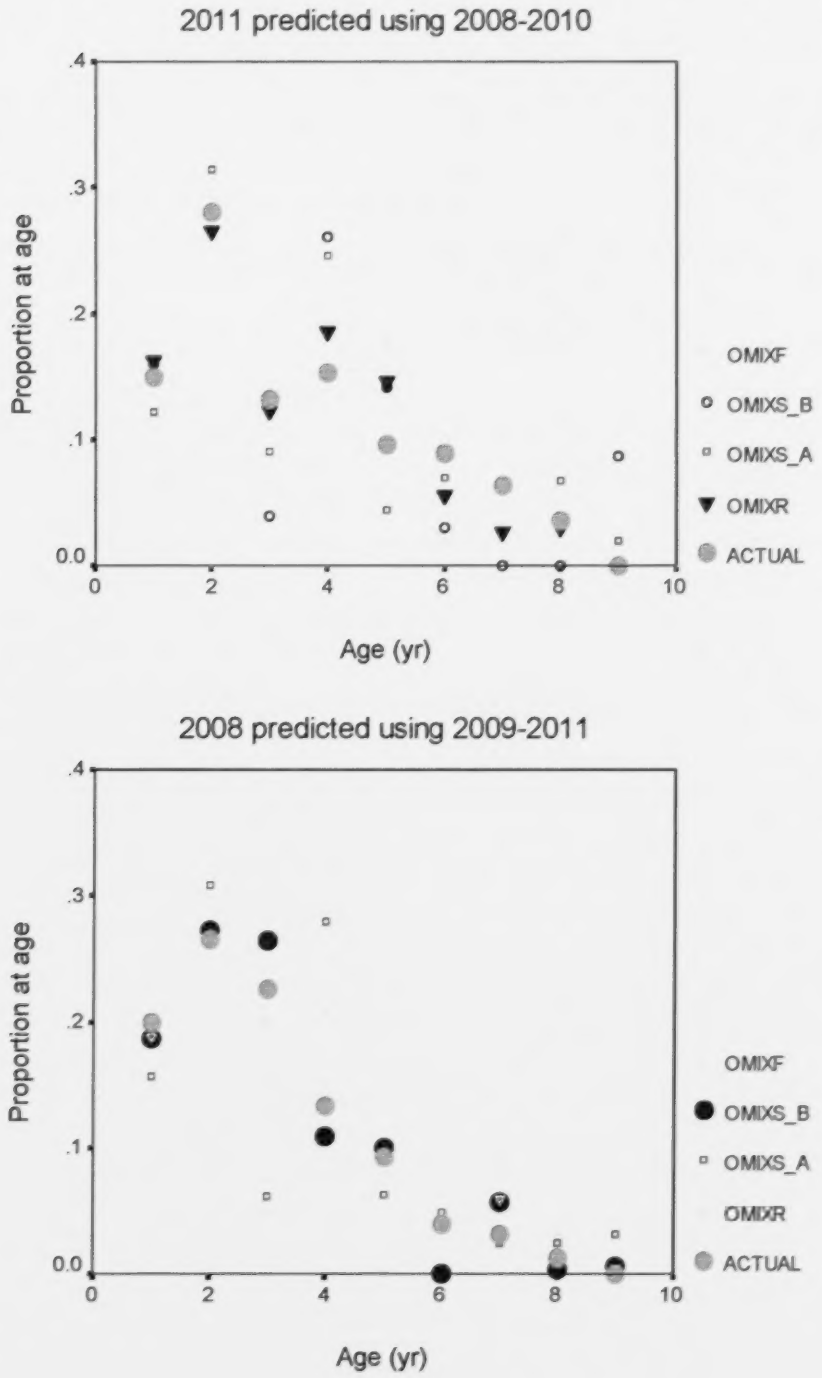

Figure 9. Proportion at age in the research vessel silver hake collection compared to estimates derived from four predictive models based on totally independent data. (Top) Age proportions in 2011 (n=281; red symbols) predicted using 2008-2010 data (n=729). (Bottom) Age proportions in 2008 (n=226; red symbols) predicted using 2009-2011 data (n=787). The model providing the most accurate estimates is indicated by dark filled symbols.

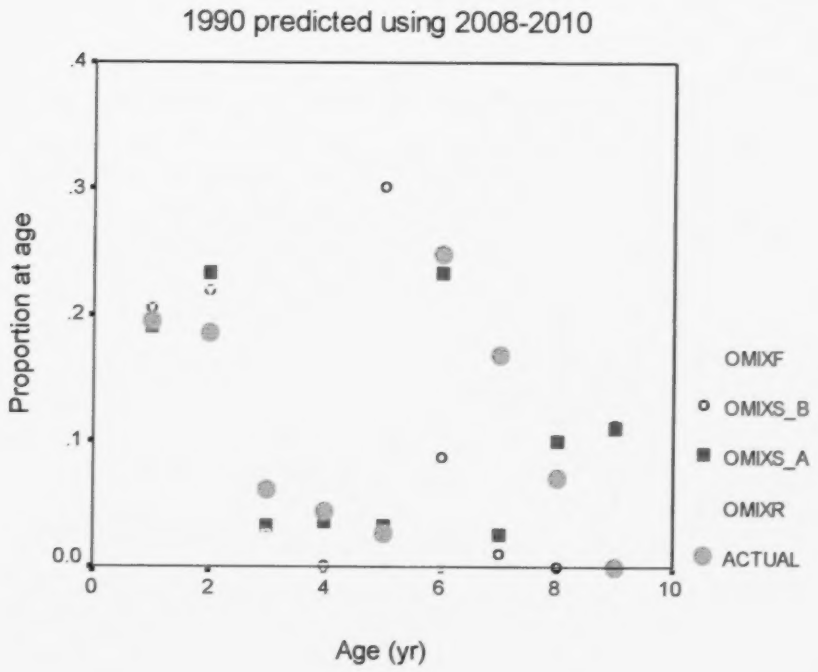

Figure 10. Proportion at age in the 1990 research vessel silver hake collection (n=113; red symbols) compared to estimates derived from four predictive models based on 2008-2010 data (n=729). Fish and otolith size at age differed significantly between 1990 and 2008-2010. The model providing the most accurate estimates is indicated by blue squares.